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Isolation and Partial Characterization of a Mycotoxin from *Penicillium roqueforti*

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Extracts of pure cultures of *Penicillium roqueforti* isolated from toxic feed samples and of *P. roqueforti* NRRL 849 were lethal to rats by either intraperitoneal or oral administration. Purification studies guided by this test led to the isolation of a major toxin which showed intraperitoneal and oral median lethal dose values in weanling rats of 11 and 115 mg/kg, respectively. Partial characterization of the crystalline compound, $C_{17}H_{20}O_6$, by infrared, ultraviolet, PMR, and mass spectroscopy, and by several chemical transformations indicated the presence of three C-methyl substituents plus one acetoxy, one aldehyde, and one α,β-unsaturated ketone group. Two oxygen atoms are present either in epoxide or ether form.

The principle microorganism isolated from ground moldy mixed grains and corn silage associated with cases of bovine abortion and placental retention in Wisconsin, was *Penicillium roqueforti* (P. E. Still et al., Fed. Proc. 31:733). Chloroform-extracted material from 200 g of dried feed was lethal to adult (250 g) rats by oral administration. Moldy corn silage samples from other Wisconsin farms were also found in which *P. roqueforti* prevailed as the predominant microorganism. Pure cultures of *P. roqueforti* isolated from the feed samples when grown either on moist whole corn or 2% yeast extract-15% sucrose broth were similarly toxic to rats.

P. roqueforti from silage and milled rice in Japan has recently been reported to produce toxic metabolites (2, 3). Kanota (2) obtained three toxins from *P. roqueforti* grown on Czapek liquid medium, one of which was partially characterized and suggested to have the formula $C_7H_{12}O_7$. No further report has appeared.

The present report describes the isolation and partial characterization of a toxic fluorescent metabolite, herein referred to as PR toxin, from *P. roqueforti* cultures.

MATERIALS AND METHODS

Organism. The strain of *P. roqueforti* isolated from toxic moldy feeds was used in the beginning for production of PR toxin. Since it was later found that *P. roqueforti* NRRL 849 produced much higher toxin yields, the latter strain was used in most of these investigations.

The fungus was grown in a culture medium containing 2% yeast extract (Difco) and 15% sucrose. One-liter Erlenmeyer flasks containing 110 ml of medium per flask were stoppered with cotton plugs and sterilized at 121 C for 17 min. Each was inoculated with about 10^6 to 10^8 spores of *P. roqueforti* washed from potato-dextrose-agar slants, and the flasks were then incubated as stationary cultures for 14 days at 24 C.

Detection of toxin. Toxic samples were chromatographed on Silica Gel G(Brinkmann Instruments Co.) thin-layer plates with methanol-chloroform (4:96, v/v). In sufficiently purified samples the fluorescence of the toxin could be detected under ultraviolet light (Mineralight UVS 11, Ultra-Violet Products, Inc., San Gabriel, Calif.) as a characteristic dark-blue spot at R_f about 0.63. The fluorescent color changed to gray-green after brief exposure to ultraviolet light and became visible in ordinary light as a yellow spot. The toxin also could be recognized on the thin-layer chromatography (TLC) plate by spraying with 50% sulfuric acid. It appeared immediately as a yellow spot which after charring at 230 C for 2 min turned yellowish-brown.

Biological assay. Weanling male albino rats (50–60 g) were used as test animals. Samples containing 1 to 2 or 10 to 20 mg of toxin, respectively, were administered intraperitoneally or orally as solutions in 0.1 to 0.2 ml of propylene glycol. Control animals were dosed in the same manner omitting the toxin.

Isolation of toxin. Three liters of culture filtrate was extracted three times with 2 liters of chloroform each time. The extracts were evaporated in vacuo to a thick brown oil (6.97 g), with further purification by passage, respectively, through three different columns.

The oil was dissolved in a small amount of chloroform, applied to a 3-cm diameter column packed with 20 g of silica gel (70 to 325 mesh), and eluted with chloroform. The first 45 ml of effluent was discarded. When a brown band began to be eluted, collection was begun. Most of the toxin was contained in the following 50-ml fraction, which was evaporated in vacuo to a brown oil (3.3 g).

The oil from the above column was then dissolved in about 5 ml of methanol-chloroform (4:96, v/v), applied to a 3.3- by 60-cm column packed with 240 g of silica gel, and eluted with the same solvent. The flow rate was adjusted to 0.5 ml per min, and 20-min fractions were collected. PR toxin, monitored by the TLC method, appeared in fractions 59 to 69 which were then combined and evaporated in vacuo at ca. 40 C for at least 1 hr. A yellowish spongy solid weighing 1.6 g was obtained.

The third column was prepared with 80 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), soaked in Skellysolve B-chloroform (40:60, v/v) overnight, and poured into a 2.2-cm diameter column. After the column had formed by gravity, it was washed with 200 ml of the same solvent. Material (400 mg) from the second silica gel column above was dissolved in 1.2 ml of chloroform, and the solution was then mixed with 0.8 ml of Skellysolve B-chloroform (40:60, v/v). The flow rate was adjusted to 0.25 ml per min, and 20-min fractions were collected. Pure PR toxin was detected by TLC in fractions 20 to 26 which were combined and concentrated in vacuo to a glassy solid that simultaneously crystallized. The colorless crystals weighed 253 mg, mp 155 to 157 C, and chromatographed as a single spot by the TLC method. Analysis (Micro-Tech Laboratories, Skokie, Ill.) revealed: found C, 63.11; H, 6.52; O, 29.20, N, <0.1. Calculated for $C_{17}H_{20}O_6$; molecular weight 320, C, 63.74; H, 6.29; O, 29.97. The mass spectrum gave a base peak at *m/e* 277 (M − 43) and a molecular ion peak at *m/e* 320 (1.2% of the base peak), confirmed also by chemical ionization mass spectrum which showed an intense peak at *m/e* 321 (M + 1).

Properties of the toxin. PR toxin is soluble in chloroform, carbon tetrachloride, methanol, ethanol, acetone, diethyl ether and dioxane; it is insoluble in Skellysolve B, water, and in both hydrochloric acid and sodium hydroxide aqueous solutions. The ultraviolet spectrum has $\lambda_{max}^{\text{ethyl alcohol}}$ at 247 nm (ϵ 8,800). However, the ethanolic solution rapidly turned yellow when exposed to ultraviolet light. The 247-nm peak simultaneously collapsed to a broad band around 244 nm and a new peak appeared at 400 nm. The fluorescence energy distribution peak of PR toxin in reagent grade methanol was at 360 nm with an excitation wavelength of 300 nm.

The infrared spectrum of PR toxin has bands at 2,945 cm^{-1} (methyl stretching); 1,735 cm^{-1}, 1,720 cm^{-1}, and 1,680 cm^{-1} (three C=O); 1,620 cm^{-1} (ethylenic unsaturation); 1,460 cm^{-1}, 1,435 cm^{-1}, and 1,380 cm^{-1} (methyl bending); 1,245 cm^{-1} and 1,035 cm^{-1} (C—O—C). The absence of any band in the region of 3,400 cm^{-1} indicated absence of free hydroxyl groups.

The PMR spectrum (Fig. 1) showed a three-proton doublet at δ1.03 (J = 7 Hz, CH_3), a three-proton doublet at δ1.45 (J = 0.8 Hz, CH_3), a three-proton singlet at δ1.49 (CH_3), a three-proton singlet at δ2.16 ($OCOCH_3$), a one-proton doublet at δ3.65 (J = 3.5 Hz), a one-proton doublet of doublets at δ3.96 (J = 3.5, 5 Hz), a one proton doublet of doublets atδ5.16 (J = 5, 5Hz), a one-proton singlet at δ6.43, (isolated ethylenic proton), and a one-proton singlet at δ9.75 (CHO). These signals account for 17 protons. The three remaining protons are spread between δ1.6 to 2.3.

Chemical reactions; alkaline hydrolysis. PR toxin (50 mg) was dissolved in a 0.02 N solution of potassium hydroxide in methanol-water (4:1, v/v) (2.5 ml) and allowed to react at 40 C for 3 hr. The reaction mixture, monitored by TLC, revealed that most of the toxin was converted to one major product having a lower R_f value (0.37) in methanol-chloroform (4:96, v/v). The reaction mixture was diluted with water and extracted with chloroform, and the chloroform layer was dried over anhydrous sodium sulfate and then evaporated to an oil (46 mg). Chromatography of the oil on a silica gel column (2 by 60 cm) developed with methanol-chloroform (4:96, v/v) gave several 4-ml fractions containing the hydrolysis product as monitored by TLC. The combined, concentrated fractions yielded a clear oil which crystallized readily (28 mg). The colorless crystals melted at 113.5 to 115 C, and showed λ_{max}^{MeOH} at 247 nm. The infrared spectrum lacked the carbonyl band at 1,735 cm^{-1} shown by the parent compound.

Sodium borohydride reduction. PR toxin (193 mg) was dissolved in 100 ml of ethanol, sodium borohydride (600 mg) was added, and the mixture was allowed to react at 40 C for 2 hr. Water (400 ml) was then added and the *p*H was adjusted to 5 with 1 N HCl. The mixture was extracted three times with 200 ml of chloroform, and the combined chloroform layers were evaporated to yield an oil (165 mg). Chromatography of the oil on a silica gel column (2 by 60 cm) developed with methanol-chloroform (10:90, v/v) gave several 4-ml fractions containing the reduced product as monitored by TLC. The appropriate fractions were combined and evaporated to a clear oil (130 mg), which gave a single TLC SPOT with R_f 0.55 in methanol-chloroform (15:85, v/v). The reduced product showed no fluorescence on TLC plates, no ultraviolet absorption in the 247-nm region, and a molecular ion peak in the mass spectrum at *m/e* 324.

Reaction with ammonium hydroxide. PR toxin (50 mg) was dissolved in a mixture of 2 ml of methanol, 0.35 ml of water, and 0.15 ml of concentrated ammonium hydroxide. Examination of the reaction mixture by TLC revealed that the toxin was converted within 5 min to a product having a lower R_f value (0.18) in methanol-chloroform (4:96, v/v). The mixture was evaporated at room temperature in a stream of nitrogen to a yellowish oil which was chromatographed on a silica gel column (2.2 by 32 cm) eluted with methanol-chloroform (4:96, v/v). Effluent fractions containing the product as in-

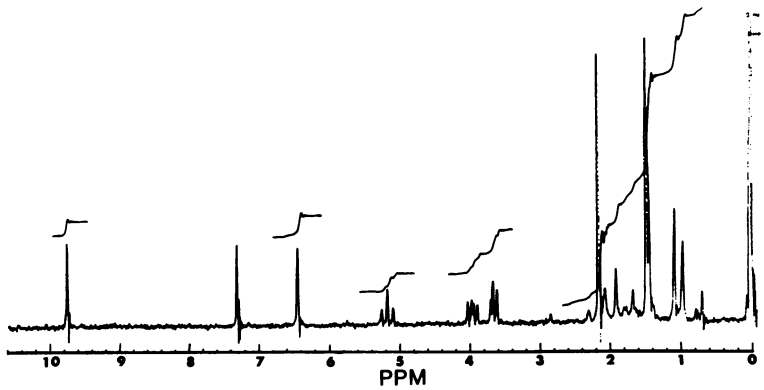

FIG. 1. *Sixty MC PMR spectra of PR toxin in $CDCl_3$ with tetramethylsilane as internal standard. Peak at δ7.28 due to $CHCl_3$.*

dicated by TLC were evaporated to an oil (35 mg). The oil was dissolved in a small volume of chloroform and crystallized by addition of carbon tetrachloride. The pale yellow crystals melted at 85 to 88 C, and showed λ_{max}^{MeOH} at 247 nm. The mass spectrum showed a molecular ion peak at *m/e* 319.

Spectroscopic measurements. Ultraviolet and visible spectra were carried out in a Beckman DB spectrophotometer equipped with 1 ml of cells. Infrared spectra were determined in a Beckman IR-5 infrared spectrophotometer. Mass spectra were taken on an Associated Electrical Industries, Ltd. MS-9 mass spectrometer. PMR spectra were measured in Varian T-60 NMR spectrometer.

All isolation work and experiments with the purified toxin were done in subdued light or in a darkened room because of the marked light sensitivity of the toxin.

RESULTS AND DISCUSSION

Production of toxin. PR toxin was mainly present in the culture medium. The *p*H of the medium when harvested was about 5.5. In our early experiments only milligram quantities of the toxin were obtained from many liters of culture medium. By careful study of culture conditions, gram quantities of the toxin are now obtainable from a few liters of culture medium. Details will be reported elsewhere.

Toxicity. The intraperitoneal and oral median lethal dose values of the pure PR toxin for weanling rats were calculated, according to the formula of Weil (4), to be 11 and 115 mg/kg, respectively. Within 10 min after ip injection of 1 to 2 mg of PR toxin to 60-g rats (16-33 mg/kg), the animals had lost the ability to support their weight. Death occurred in a few hours to a few days with excessive amounts of edematous fluid and fibrin in the intraperitoneal cavity. In lower-dosed animals (0.25 mg to 0.5 mg per rat) that survived for several weeks, adhesions of the abdominal organs were observed. Within 10 min after an oral dose of about 10 mg (160 mg/kg), the animals developed breathing difficulties which persisted until death. Oral doses above about 130 to 160 mg/kg of body weight were fatal to 60-g rats in 36 hr or less without evidence of gross pathology except for swollen, gas-filled stomach and intestines. Histological changes included conjestion and edema of lung, brain, and kidney, and degenerative changes in liver and kidney with hemorrhage in the latter.

Partial characterization. Elemental analysis and spectral data establish the molecular formula of PR toxin to be $C_{17}H_{20}O_6$. Functional groups and structural features indicated by ultraviolet, infrared, PMR, and mass spectra and confirmed by hydrolysis, borohydride reduction, and Schiff base formation with ammonia include three methyl groups, an α,β-unsaturated carbonyl, an aldehyde, and an acetoxy group, but no free hydroxyl groups in the original molecule.

Disappearance of the three-proton singlet at δ2.16 on alkaline hydrolysis indicated removal of the acetyl group, confirmed by the mass spectrum which showed a molecular ion of *m/e* 278 and loss of the infrared band at 1,735 cm^{-1}. The newly generated secondary hydroxyl proton, at δ1.8, was exchangeable with D_2O. All other protons were unchanged after hydrolysis except that the one-proton doublet of doublets at δ5.16 was shifted up field to δ4.1, indicating that this proton is attached to the same carbon as the acetoxy group. This was confirmed by further oxidation of the secondary alcohol to a ketone (molecular weight 276) with dipyridine-chromium (VI) oxide (1). In the PMR spectrum of this ketone, the one-proton doublet of doublets at δ5.16 was absent and the one-proton doublet of doublets at δ3.96 had become a

doublet. This indicates that the protons at δ5.16 and 3.96 are on adjacent carbons in the original toxin.

PR toxin was reduced by sodium borohydride to a product of molecular weight 324 (mass spectrum) indicating that two carbonyl groups (one aldehyde and one ketone) were reduced. The PMR spectrum showed no aldehyde proton at δ9.75. The four added protons and the original aldehyde proton appeared between δ1.6 to 4.33. Since the isolated ethylenic proton at δ6.43 was shifted to δ6.0 and converted to a doublet (J = 3.5 Hz), the double bond was originally conjugated with the ketone. Saponification of the borohydride reduction product to remove the acetyl group gave a compound which according to the infrared spectrum contained no carbonyl groups. Therefore, the original toxin contained no lactone structure, and the two unassigned oxygens are probably present in ether or epoxide form.

Ammonia reacted readily with the PR aldehyde to form a Schiff base of molecular weight 319. The PMR spectrum of this product showed that the aldehyde proton at δ9.75 had disappeared with generation of two one-proton singlets at δ5.53 and 5.06. The latter was exchangeable with D_2O.

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